

## Monitoring differences in gene expression levels and polyhydroxyalkanoate (PHA) production in *Pseudomonas putida* KT2440 grown on different carbon sources

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***Pseudomonas putida* has a variety of potential uses in bioremediation and biosynthesis of biodegradable plastics. *P. putida* is able to utilize a wide range of carbon sources. In this study, *P. putida* KT2440 was grown on glucose, glycerol, citrate, or fatty acid (lauric acid) as the sole carbon source. Differences in expression levels of genes involved in the Entner–Doudoroff pathway, glycerol metabolism, TCA cycle and  $\beta$ -oxidation were detected using quantitative real-time PCR. When glycerol was the sole carbon source, expression of genes related to glycerol metabolism was enhanced with the exception of the negative regulon gene *glpR*. There were no significant differences in expression levels of genes that putatively encode enzymes involved in the Entner–Doudoroff pathway for cells grown on glucose as compared to cells grown on other carbon sources. Exceptions to this trend were the ABC transporter genes. Genes encoding enzymes selected from the TCA cycle all showed higher expression levels in cells grown on citrate. Two genes for  $\beta$ -oxidation enzymes, *fadB* and the long-chain fatty acid transporter gene, showed higher expression level when cells were grown on lauric acid. Genes encoding enzymes involved in PHA synthesis, *phaC1*, *phaC2*, *phaZ*, and *phaJ4*, all showed higher expression levels when cells were grown on lauric acid. This study has identified genes involved in the metabolism of different carbon sources and PHA synthesis. This information will be invaluable to understand how genes are regulated and construct transgenic strains to utilize carbon sources more efficiently and better produce PHAs.**

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**[Key words:** *Pseudomonas putida* KT2440; Polyhydroxyalkanoates (PHAs); Gene expression; Quantitative real-time PCR; Carbon metabolism]

Polyhydroxyalkanoates (PHAs) have attracted extensive interest as environmentally friendly biodegradable plastics. These materials are produced by many bacteria when they are grown under nutrient limitation and serve as carbon and energy storage reserve (1). Medium-chain-length (MCL)-PHAs are a subclass of these biodegradable polymers, which contain 6 to 14 carbons in their monomers (2), have elastomeric properties and can be potentially used as materials for drug delivery (3) and tissue engineering (4). Although short-chain-length (SCL)-PHAs have been commercially developed, efficient production of MCL-PHAs still remains difficult (5). *Pseudomonas putida* strains are known to produce large amounts of MCL-PHAs under nutrient limitation (6). *P. putida* KT 2440 is the best characterized strain and its genome has been fully sequenced (7). This strain is metabolically versatile and is able to utilize a wide range of carbon sources including: glycerol, sugars (such as glucose), small organic acids (such as citrate), and fatty acids (8). Examination of the genome reveals a number of genes that putatively encode enzymes for the catabolism of carbon and PHA biosynthesis. However, there is still a dearth of information regarding the regulation of the catabolic pathways and PHA biosynthesis pathways in pseudomonads. There have been few systematic studies focusing on comparing the differences of PHA production and gene

expression levels in related pathways in *P. putida* when the cells are grown on different carbon sources. Knowledge regarding the key enzymes for these pathways may prove essential to engineer *P. putida* for optimal PHA production.

In this study, the gene expression levels of key enzymes and transporters involved in the metabolism of four different carbon sources, glycerol, glucose, citrate and fatty acid (lauric acid) and PHA production (Fig. 1) in *P. putida* KT2440 were monitored by quantitative real-time PCR (QRT-PCR). We chose these four basic carbon sources to analyze pathways that would most likely be utilized by the strain for the production of bioproducts. This study is the first to systematically compare the growth and gene expression levels of *P. putida* grown on different carbon sources. This information will ultimately be useful to understand the intricacies of carbon metabolism in *P. putida* and to provide new target genes for genetic engineering in order to better utilize this versatile strain for the production of PHAs.

### MATERIALS AND METHODS

**Bacterial strains and cultivation conditions** The strain *P. putida* KT2440 was grown on a nutrient-rich growth medium (Luria broth, LB) plate at 30 °C overnight. A single colony was picked and used to inoculate 50 ml LB medium for growth overnight in a shaking incubator (150 rpm) at 30 °C. A total volume of 1 ml of this starter culture was used to inoculate 100 ml of a defined high nitrogen mineral salts (MS) medium, consisting of 3.8 g Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O, 2.65 g KH<sub>2</sub>PO<sub>4</sub>, 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 7.2 g NH<sub>4</sub>Cl and 1 ml trace element solution per liter. The trace element solution consisted of 9.7 g FeCl<sub>3</sub>,

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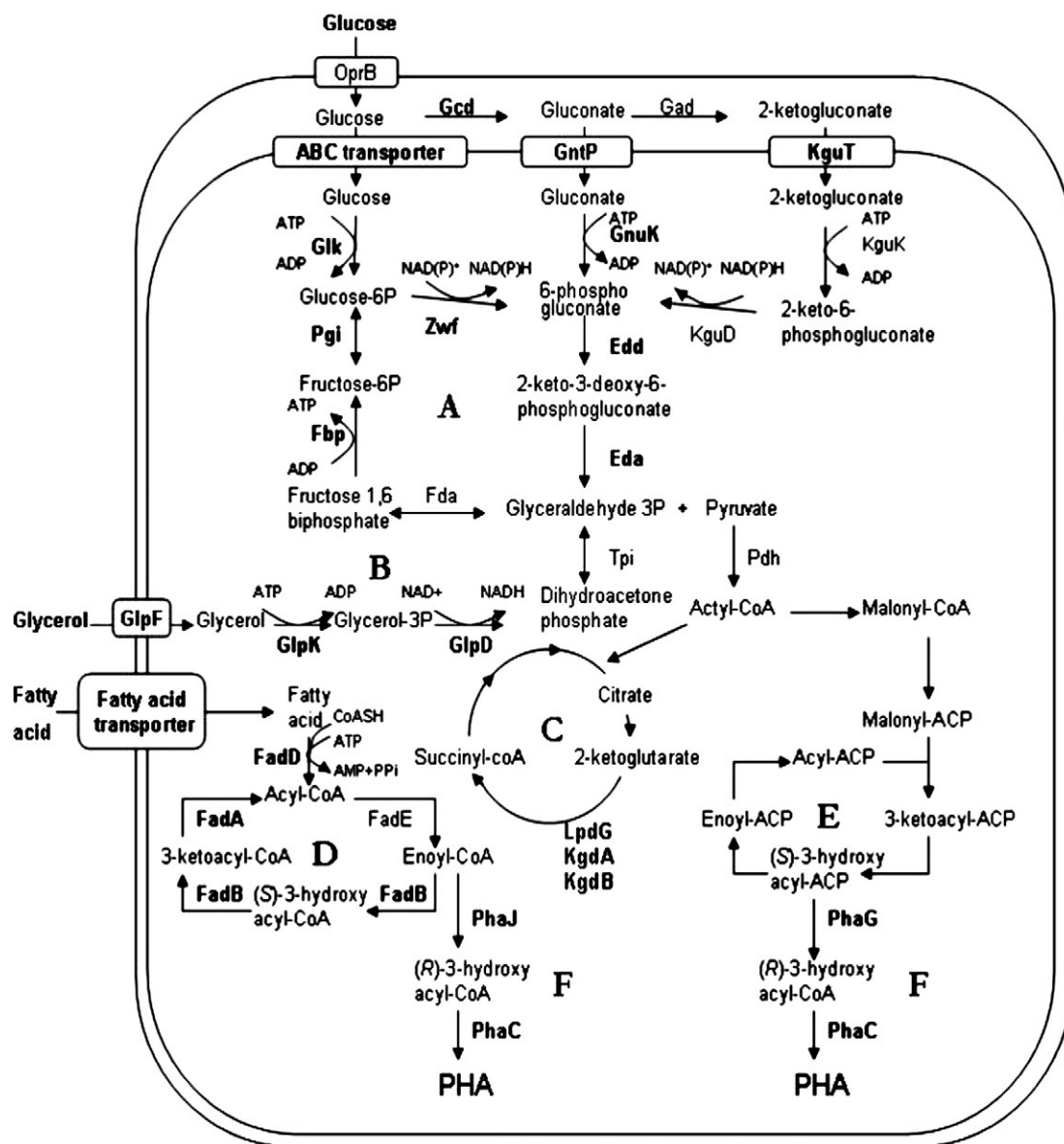


FIG. 1. Proposed pathways for metabolism of glucose, glycerol, citrate, fatty acids and PHA production in *P. putida* KT2440. A. Entner–Doudoroff pathway. B. Glycerol metabolism. C. TCA cycle. D.  $\beta$ -oxidation. E. Fatty acid biosynthesis. F. PHA biosynthesis. GlpF, glycerol uptake facilitator protein; GlpK, glycerol kinase; GlpD, glycerol-3-phosphate dehydrogenase; GlpR, glycerol-3-phosphate regulon repressor; Glk, glucokinase; Gcd, glucose dehydrogenase; GnuK, gluconokinase; Pgi, glucose-6-phosphate isomerase; Zwf, glucose-6-phosphate 1-dehydrogenase; Edd, phosphogluconate dehydratase; Eda, 2-dehydro-3-deoxyphosphogluconate aldolase; GntP, gluconate transporter; KguT, 2-ketogluconate transporter, putative; LpdG, 2-oxoglutarate dehydrogenase, lipoamide dehydrogenase component; KgdB, 2-oxoglutarate dehydrogenase, E1 component; Ctrns, citrate transporter; FadD, long-chain-fatty-acid-CoA ligase; FadB, fatty oxidation complex, alpha subunit; FadA, 3-oxoacyl-CoA thiolase; Long trans, long-chain fatty acid transporter, putative; Short trans, transporter, short-chain fatty acid transporter family; PhaC, poly(3-hydroxyalkanoate) polymerase; PhaZ, poly(3-hydroxyalkanoate) depolymerase; PhaG, acyl-transferase; PhaJ, enoyl-CoA hydratase, R-specific. The genes monitored by QRT-PCR were bolded.

7.8 g CaCl<sub>2</sub>, 0.218 g CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.156 g CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.118 g NiCl<sub>2</sub>·6H<sub>2</sub>O and 0.105 g CrCl<sub>3</sub>·6H<sub>2</sub>O per liter (9). Glucose, glycerol, citrate or the fatty acid, lauric acid, was added as the sole carbon source to the MS media to final concentrations of 20 g l<sup>-1</sup>. The experiments were performed in triplicate. Growth was assessed spectrophotometrically by measuring the optical densities at 450 nm (OD<sub>450</sub>) at 0 h, 4 h, 8 h, 12 h, 24 h, 48 h and 72 h.

**Isolation total RNA and synthesis of cDNA** A total volume of 1 ml of cell culture was harvested from MS medium after OD<sub>450</sub> reached ~5.0. RNAs were stabilized using RNAprotect Bacteria Reagent (Qiagen, USA). The total RNA was isolated using RNeasy Mini Kit (Qiagen, USA). Prior to reverse transcription, the trace DNA remaining in RNA samples was removed by digestion with RQ1 RNase-Free DNase (Promega, USA). The concentration of RNA was then measured by spectrophotometrically using a NanoDrop Spectrophotometer (Thermo, USA). The cDNA libraries were synthesized

with a SuperScript VILO cDNA Synthesis Kit (Invitrogen, USA). Total RNA (2  $\mu$ g) was added into the reaction mixture as the template for each sample. The conditions for the reverse transcription reactions were: 10 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C. All procedures using kits were performed according to the manufacturer's instructions. The experiments were performed in triplicate.

#### Determination of gene expression levels by quantitative real-time PCR (QRT-PCR)

The sequences of genes monitored by QRT-PCR were found in the database of The Institute of Genomic Research (TIGR) (<http://www.tigr.org>). Oligonucleotide primers for QRT-PCR reaction were designed using Primer 3 (<http://frodo.wi.mit.edu/primer3/input.htm>) and are listed in Table 1. The QRT-PCR reactions were performed using the iQ SYBR Green Supermix Kit with iQ5 multicolor real-time PCR detection system (Bio-Rad, USA). The reaction conditions were 2 min at 94 °C, 40 × (40 s at 94 °C, 40 s at 53 °C, and 40 s at 68 °C), and 8 min at 68 °C, followed by melting curve analysis: 1 min at 95 °C, 81 × (30 s starting at

TABLE 1. Primers used in QRT-PCR.

| Gene name                                   | Locus name | Oligonucleotide sequence    |                             |
|---|------------|-----------------------------|-----------------------------|
| <i>Genes related to glycerol metabolism</i> |            |                             |                             |
| <i>glpF</i>                                 | PP 1076    | 5'-AGCATGGCCATCTACCTCAC-3'  | 5'-ATACCTGGGCCAGCATGTAG-3'  |
| <i>glpK</i>                                 | PP 1075    | 5'-AAGGTCAAGGACAGCAATGG-3'  | 5'-GCGGATGATGGTCTACCT-3'    |
| <i>glpD</i>                                 | PP 1073    | 5'-GTGACCCGGACTACACT-3'     | 5'-GCGGATGATGGTCTACCT-3'    |
| <i>glpR</i>                                 | PP 1074    | 5'-AGGCCATGTGCGATTTCATC-3'  | 5'-ACACCCGCATCTCTGATAG-3'   |
| <i>Genes in Entner–Doudoroff pathway</i>    |            |                             |                             |
| <i>glk</i>                                  | PP 1011    | 5'-ATATGTGGGCGGTGATT-3'     | 5'-CCGTGAAATAGCCACTCAT-3'   |
| <i>gcd</i>                                  | PP 1444    | 5'-GACGAAGACCTTGGCATGAT-3'  | 5'-TCGTGGTGGGTGAAGTATA-3'   |
| <i>gnuK</i>                                 | PP 3416    | 5'-AAAACATCCGCAAGATGAGC-3'  | 5'-GTTCCGAGAACACGAACACC-3'  |
| <i>pgi-1</i>                                | PP 1808    | 5'-CACCTCAGGATGCTACTGCT-3'  | 5'-CAAGCGCTAAGGAAAAGT-3'    |
| <i>pgi-2</i>                                | PP 4701    | 5'-ACCACCTGTTTCATCTCTC-3'   | 5'-GCTGATACGGCAATGAAGT-3'   |
| <i>zwf-1</i>                                | PP 1022    | 5'-CCCATTGCAGTGAATTTT-3'    | 5'-CCAGGCATACTCGAATTCGT-3'  |
| <i>zwf-2</i>                                | PP 4042    | 5'-CCCAAACCACTACTGAA-3'     | 5'-GCAAAACCACTCGACATT-3'    |
| <i>zwf-3</i>                                | PP 5351    | 5'-CCACGATCGACTATCTGAA-3'   | 5'-GAAGCATCCATCGGGAAGTA-3'  |
| <i>edd</i>                                  | PP 1010    | 5'-TGATTCGTGAAGTCTCGAC-3'   | 5'-GCGACGGCAGATACTTTCAT-3'  |
| <i>eda</i>                                  | PP 1024    | 5'-CATTGCTCGTGAGGAAGACA-3'  | 5'-CCTCGAGAATGTCCTGGTA-3'   |
| <i>ABC-1</i>                                | PP 1015    | 5'-AAGTCGCCACTACATGGAC-3'   | 5'-AGTCTCACCCAACACCTTG-3'   |
| <i>ABC-4</i>                                | PP 1081    | 5'-ACACCCTCAACCTGCAATT-3'   | 5'-AGTGAGCCAGTGTCTTCT-3'    |
| <i>gntP</i>                                 | PP 3417    | 5'-GACCATCTTCTACGGCCTGA-3'  | 5'-GAACGTTTTCAGCAGATCA-3'   |
| <i>kguT</i>                                 | PP 3377    | 5'-CTGGTGTACCTGTGCCAATTG-3' | 5'-ACAGGCTTGATACCTCTGCTG-3' |
| <i>Genes in TCA cycle</i>                   |            |                             |                             |
| <i>lpdG</i>                                 | PP 4187    | 5'-AGAAGCTCATCGTCACTCC-3'   | 5'-GGCGTTGGTGTAGTCACTT-3'   |
| <i>kgdB</i>                                 | PP4188     | 5'-GTCACCAAGGAAGACGTGGT-3'  | 5'-CACTTCGTGAAGTGGTCA-3'    |
| <i>kgdA</i>                                 | PP 4189    | 5'-TCCGGTGACGTGAAGTATCA-3'  | 5'-GGTTGTTGATCAGGATGTGC-3'  |
| <i>ctrans</i>                               | PP 0147    | 5'-GCCTTCAGTATCGCCATGAT-3'  | 5'-CGTTGGACATGAAGAACGTG-3'  |
| <i>ABC</i>                                  | PP 0171    | 5'-GAAGAGCTGTTGCTGTG-3'     | 5'-TCCGGGATATCTGCTTCATC-3'  |
| <i>Genes in beta-oxidation</i>              |            |                             |                             |
| <i>fad D</i>                                | PP 4549    | 5'-ATGCGTTTACCTTCCATTGC-3'  | 5'-CTTAGTGAAGGCCATACC-3'    |
| <i>fadD2</i>                                | PP 4550    | 5'-GCAGAAGGCTGGTCAAGAC-3'   | 5'-GGGTACTTTGTAGCCGGTGA-3'  |
| <i>fadDx</i>                                | PP 2213    | 5'-TTTACCAGCCCTTGTTCACC-3'  | 5'-TGAACATCAGCAGGAATGGA-3'  |
| <i>fadB</i>                                 | PP 2136    | 5'-GCCTACTTGATGACGTGGT-3'   | 5'-TCAGTACTTACCGCTGCTC-3'   |
| <i>fadBx</i>                                | PP 2214    | 5'-CATCAACGTCACCTGATCG-3'   | 5'-AAAGATACCCGGACGGATG-3'   |
| <i>long trans</i>                           | PP 1689    | 5'-GCCAGCCTGAAGATCAAGAC-3'  | 5'-CCTTGATCAGATCACCATG-3'   |
| <i>short trans</i>                          | PP 3124    | 5'-GTGTTTGAGGTTTGTGGT-3'    | 5'-GCCAGAAAAGATGGTTTCG-3'   |
| <i>Genes related to PHA synthesis</i>       |            |                             |                             |
| <i>phaC1</i>                                | PP 5003    | 5'-CAGGTGCTTTGTTTGTG-3'     | 5'-TTGTTTACCAGTAGTTCC-3'    |
| <i>phaZ</i>                                 | PP 5004    | 5'-AGTTTGCTCAGATTAC-3'      | 5'-CACCTGGGCTTGC-3'         |
| <i>phaC2</i>                                | PP 5005    | 5'-ATGAGCAGACCATCG-3'       | 5'-GTTTACCAGTAGTTCC-3'      |
| <i>phaG</i>                                 | PP 1408    | 5'-TTCAAACGCTTCAAC-3'       | 5'-CGGTCTTGTCTCC-3'         |
| <i>phaJ</i>                                 | PP 4552    | 5'-TGTCCCAGGTGAC-3'         | 5'-GGAACATGCTCTTG-3'        |
| <i>phaJ3</i>                                | PP 0580    | 5'-CCGAGGCTGAAGATGGTATC-3'  | 5'-ACCGCACTGAGTGGATAGG-3'   |
| <i>phaJ4</i>                                | PP 4817    | 5'-ATGGTTTCTGACCTGTGCG-3'   | 5'-AAAAACAGAGCCAGCCGACT-3'  |
| <i>House keeping gene</i>                   |            |                             |                             |
| <i>rpoD</i>                                 | PP 0387    | 5'-AGCCGACTTCT-3'           | 5'-AGCCGACTTCTG-3'          |

55 °C, increasing 0.5 °C per cycle, ending at 95 °C). The experiments were performed in triplicate. The gene expression levels were assessed by the following formula:

$$\text{Gene expression level} = 2^{\text{Ct}(rpoD) - \text{Ct}(\text{target})}$$

Ct values represent the first cycle at which the instrument can distinguish the fluorescence of nucleic acid amplification generated as being above the background signal. This method compares the Ct value of the house keeping gene *rpoD* (10) to the Ct value of the target gene.

**GC analysis of PHA composition** Liquid cultures (100 ml) were centrifuged at 5000 ×g for 10 min at 4 °C. The cells grown on glucose, glycerol and citrate were washed by 10 ml Mili-Q water, and the cells grown on lauric acid were washed by 10 ml methanol to remove lauric acid (11). Finally the cells were resuspended by 1 ml Mili-Q water, and lyophilized for a minimum of 24 h. The cell dry weight was measured. The dry cells (~15 mg) were treated with 2 ml of methanol-sulfuric acid (85:15) solution and 2 ml chloroform at 100 °C for 140 min. After cooling to room temperature, the water-soluble contents were removed by washing with 1 ml Mili-Q water. The aqueous and organic phase were allowed to separate, and the organic phase containing chloroform-soluble methyl esters was taken and filtered through an acrodisc syringe filter with 0.45-µm polytetrafluorethylene (PTFE) membrane (Pall). A total of 0.5 ml chloroform mixture containing the soluble methyl ester and 0.5 ml 0.1% caprylic acid in chloroform were mixed well, and these samples were assayed using GC 2010 Gas Chromatograph (Shimadzu, Japan) (9,12). The experiments were performed in triplicate.

**Statistics** The expression levels of genes are presented as a mean value of three replicates. One way ANOVA was performed and multiple comparison was made by Dunnett's t tests at a significance level of 0.05 (overall experiment-wise error rate).

## RESULTS

**Growth rates of *P. putida* KT2440 on different carbon sources** The growth of *P. putida* KT2440 grown on glucose, glycerol, citrate or the fatty acid, lauric acid as the sole carbon source were measured at different time points (Fig. 2). The growth of cells on citrate was the fastest in the first 24 h, and the doubling time was 1.63 h during the exponential phase. But the cells began to aggregate and could not be measured by spectrometer at 48 h. The cells grown on glycerol showed a significant lag phase in the first 12 h, but they started to grow rapidly after 12 h. Their doubling time was 1.65 h during the exponential phase, and their OD<sub>450</sub> at 48 h was higher than those of cells grown on glucose or lauric acid. The doubling time during the exponential time were 1.69 h for both cells grown on glucose and lauric acid, but the OD<sub>450</sub> at 48 h of cells grown on lauric acid was higher than that of cells grown on glucose.

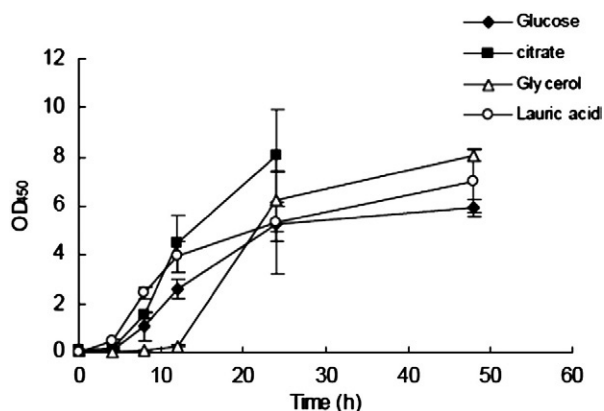


FIG. 2. Growth curves of *P. putida* KT2440 grown on different carbon sources. Results are the averages  $\pm$  standard deviations from three separate experiments.

**Expression levels of glycerol metabolic genes** The expression levels of four genes (*glpF*, *glpK*, *glpD* and *glpR*) involved in glycerol metabolism from cells grown on the glucose, glycerol, citrate or lauric acid were monitored by QRT-PCR (Fig. 3). As expected, the gene expression levels of *glpF*, *glpK*, and *glpD* were about 10-fold higher in cells grown on glycerol as the sole carbon source than those in cells grown on glucose or citrate, and about 5-fold higher than those in cells grown on lauric acid (Fig. 3). However, there were no significant differences ( $P > 0.05$ ) in expression levels of the negative repressor gene *glpR* when cells were grown on different carbon sources.

**Expression levels of genes from the Entner–Doudoroff (ED) pathway** The expression levels of genes of the key enzymes in the ED pathway, *gcd*, *glk*, *gnuk*, *pgi*, *zwf*, *edd* and *eda* were monitored by QRT-PCR. An ABC glucose transporter, which is encoded by four genes (*ABC1*, *ABC2*, *ABC3*, and *ABC4*) in an operon putatively transfers glucose into the cell. The expression levels of two (*ABC1* and *ABC4*) of the four genes encoding the putative ABC glucose transport protein were selected to be monitored. Gluconate is transferred into the cell by *GntP*, and 2-ketogluconate is transferred by *KguT* (13). The expression levels of both these transporter genes were also monitored by QRT-PCR. Surprisingly, all the genes involved in the ED pathway did not show the highest expression levels when cells were grown on glucose (Fig. 4A and B). The expression level of *gnuk* was higher in cells grown on glucose than that in cells grown on glycerol and citrate, and similar to that in cells grown on lauric acid. The expression levels of *zwf-1* and *eda* were higher in cells grown on glucose than those in cells grown on citrate, but lower than those in cells grown on glycerol. The expression level of *glk* showed no significant difference ( $P > 0.05$ ) among cells grown on different carbon sources. The expression levels of genes, *gcd*, *pgi-1*, *pgi-2*, *zwf-2* and

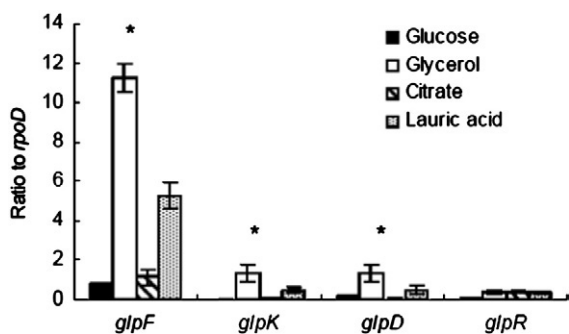


FIG. 3. Expression levels of glycerol metabolic genes from cells grown on different carbon sources. Results are the averages  $\pm$  standard deviations from three separate experiments. Asterisks indicate that the expression levels of cells grown on glycerol have significant differences ( $P < 0.05$ ) from the other carbon sources.

*zwf-3* were lower in cells grown on glucose than those in cells grown on other carbon sources. However, the expression levels of all the transporter genes were higher in cells grown on glucose than those in cells grown on other carbon sources. The gene expression levels of ABC transporter genes were at least 6-fold higher, and the gene expression levels of *gntP* and *kguT* were about two-fold higher.

**Expression levels of genes in the TCA cycle** The expression levels of genes of three enzymes, *LpdG*, *KgdB* and *KdgA*, which catalyzed one of the rate-limiting steps of the TCA cycle, the citrate transporter (*Ctrans*) and an ABC transporter which was putatively involved in citrate transport were monitored. The expression levels of gene *lpdG*, *kgdB* and *kdgA* were at least 10-fold higher in cells grown on citrate than those of cells grown on glucose, at least 4-fold higher than those of cells grown on glycerol and at least 1.2-fold higher than those of cells grown on lauric acid (Fig. 5). The expression levels of *ctrans* were about 3-fold higher, and *ABC* were at least 10-fold higher when cells were grown on citrate. All the genes selected in the TCA cycle and transporter genes seemed to be triggered by citrate.

**Expression levels of genes in  $\beta$ -oxidation** *In silico* analysis of the *P. putida* genome revealed three types of acyl-CoA synthetase genes, *fadD*, *fadDx* and *fadD2*, two types of FadBA genes, *fadA*, *fadB* and *fadAx*, *fadBx*, and the two fatty acid transporter genes, long-chain fatty acid transporter (*long trans*) and short-chain fatty acid transporter (*short trans*). The genes *fadA* and *fadB* are on the same operon, and *fadAx* and *fadBx* are on another operon. Since expression levels should be similar for genes on same operon, just *fadB* and *fadBx* were selected for QRT-PCR. The expression levels of genes *fadB* and *long trans* were at least 10-fold higher when cells were grown on lauric acid as the sole carbon source. None of the three *fadD* genes exhibits the highest expression levels in cells grown on lauric acid compared to cells grown on different carbon sources (Fig. 6).

**PHA production and expression levels of PHA synthesis related genes** Only cells grown on the fatty acid dodecanoate (lauric acid) could synthesize high amounts of PHAs without nitrogen limitation.

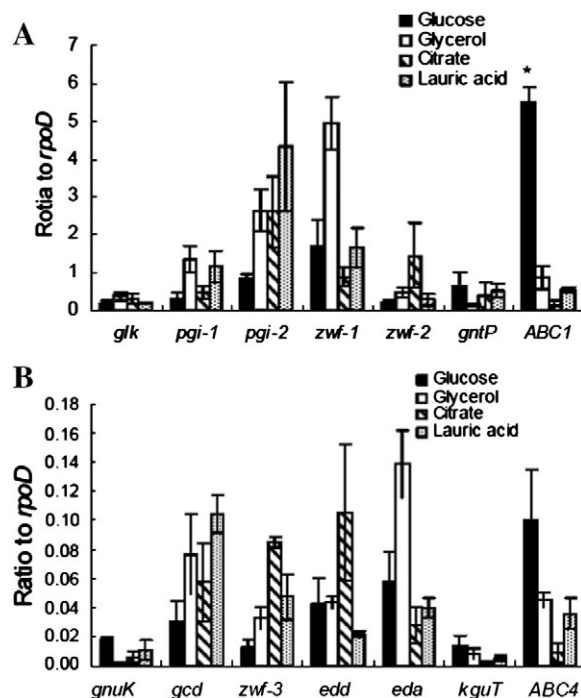


FIG. 4. Expression levels of glucose metabolic genes from cells grown on different carbon sources. Results are the averages  $\pm$  standard deviations from three separate experiments. Asterisks indicate that the expression levels of cells grown on glucose have significant differences ( $P < 0.05$ ) from the other carbon sources.

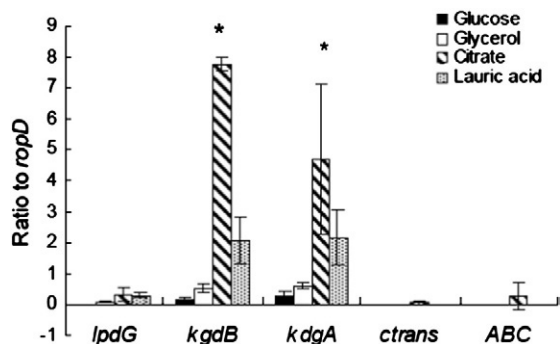


FIG. 5. Expression levels of genes in TCA cycle from cells grown on different carbon sources. Results are the averages  $\pm$  standard deviations from three separate experiments. Asterisks indicate that the expression levels of cells grown on citrate have significant differences ( $P < 0.05$ ) from the other carbon sources.

PHA production from cells grown on lauric acid was about 10-fold higher than those from cells grown on other carbon sources, and the major monomer was 3HO (about 60%) (Table 2).

Four *phaJ* genes have been found in *Pseudomonas aeruginosa* (14). Based on a database search of the genomic DNA of *P. putida* KT2440, three homologs of *phaJ*<sub>pa</sub>, *phaJ*<sub>3pa</sub> and *phaJ*<sub>4pa</sub> were found and named *phaJ1*, *phaJ3* and *phaJ4*. The gene expression levels of *phaC1*, *phaC2*, and *phaZ* were at least 15-fold higher in cells grown on lauric acid than those in cells grown on glucose, about 4-fold higher than those in cells grown on glycerol and citrate (Fig. 7). The gene expression levels of *phaJ4* were 6-fold higher in cells grown on lauric acid than those of cells grown on glucose, and 20-fold higher than those in cells grown on glycerol, and 4-fold higher than those in cells grown on citrate. There were no significant differences ( $P > 0.05$ ) in gene expression levels of *phaG*, *phaJ1* and *phaJ3*. The fact that the *phaJ1* and *phaJ3* genes were not expressed during PHA synthesis suggested these two genes unlikely to be involved in PHA production under the conditions tested.

## DISCUSSION

**Glycerol metabolism in *P. putida*** Previous research suggests that the *glp* regulon, which is involved in glycerol metabolism in gram-negative bacteria, shares a common regulatory theme (15). The expression levels of *glpF*, *glpK* and *glpD* were induced by glycerol (Fig. 3). The inducer for the *glp* regulon is *sn*-glycerol-3-phosphate (G3P) in both *E. coli* and *P. aeruginosa* (15,16). Since G3P is the intermediate of glycerol metabolism, the actual inducer of *glp* regulon is likely to be G3P in *P. putida*. The *glpF* and *glpK* genes are in the same operon in *E. coli* (17). The expression patterns of *glpF* and *glpK* in *P.*

*putida* were similar, so the statement that *glpF* and *glpK* are in the same operon in *P. putida* was confirmed in this study. The *glpR* gene is in a different operon (16). Since there were no significant differences ( $P > 0.05$ ) in gene expression levels of *glpR* in cells grown on different carbon sources (Fig. 3), it seemed that the expression levels of *glpR* were not affected by glycerol. The fact that the expression levels of *glpF*, *glpK* and *glpD* were significantly higher ( $P < 0.05$ ) when cells were grown on glycerol (Fig. 2) suggested that all enzymes involved in glycerol metabolism were induced by glycerol. This may explain the long lag phase we observed in the growth of cells on glycerol (Fig. 2), because cells needed this time to induce the expression of the Glp enzymes for the efficient utilization of glycerol.

**Glucose metabolism in *P. putida*** It has long been postulated that *Pseudomonas* species use the Entner-Doudoroff pathway for the catabolism of glucose. For genes identified by homology to be involved in glucose metabolism, the expression levels were surprisingly low compared to those of cells grown on other carbon sources (Fig. 4A and B). The exceptions to this trend were the expression levels of putative glucose transporter genes (Fig. 4A and B). The higher expression levels of *zwf*, *edd*, *eda* and *pgi* from cells grown on other carbon sources over glucose might be due to the production of NADPH, which is an important cofactor for biosynthesis, oxidation and reduction reactions in the cells. The conversion of glucose-6-phosphate to 6-phosphogluconate catalyzed by glucose-6-phosphate dehydrogenase is an important reaction to generate NADPH. This enzyme is putatively encoded by *zwf-1*, *zwf-2* and *zwf-3* in *P. putida*, and the expression level of *zwf-1* is consistent with previous study of the effect of carbon sources on *zwf* transcription on *P. aeruginosa* (18).

Previous research compared gene expression levels of cells grown on glucose and citrate as the sole carbon source via gene chip, and the results showed that expression levels of *gcd*, *glk*, *gnuK*, *zwf-1*, *edd* and *eda* were higher in cells grown on glucose than those in cells grown on citrate (13). In this study, only genes *gnuK*, *zwf-1* and *eda* showed higher expression levels in cells grown on glucose compared to cells grown on citrate. These differences might be due to the sensitivity of the different techniques. QRT-PCR is considered to be more sensitive and accurate compared to gene chip analysis, and usually used for validation of results achieved by gene chip (19). However, these differences may have also arisen since the cells were harvested at different times, and the presence of mRNA species might change very rapidly over a short time. Previous research also found that the enzyme activities of Glk and GnuK were 10-fold higher in cells grown on glucose than in cells grown on citrate (13). Based on our results, the increase of gene expression levels of these two enzymes was unlikely to be the reason for the enhancement of enzyme activities. An enzymatic study showed that the reaction rate of Glk increased when glucose was added (20), so the reason for the enhancement of the enzymatic activity in cells grown on glucose is likely due to an activation of the enzyme rather than transcript level. Genome

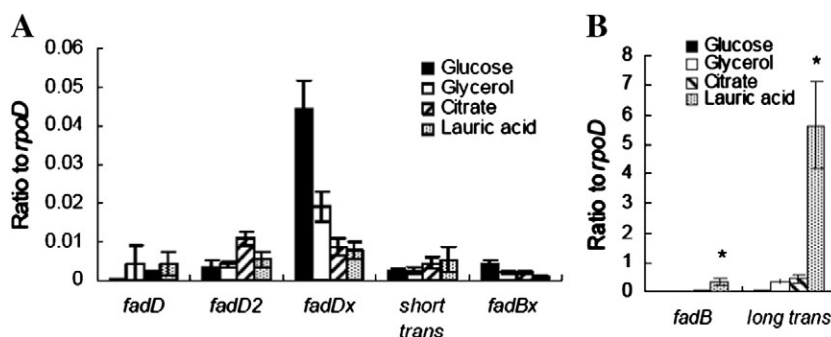


FIG. 6. Expression levels of genes in beta-oxidation from cells grown on different carbon sources. Results are the averages  $\pm$  standard deviations from three separate experiments. Asterisks indicate that the expression levels of cells grown on fatty acid have significant differences ( $P < 0.05$ ) from the other carbon sources.

**TABLE 2.** PHA accumulation in *P. putida* KT 2440 strain grown on different carbon sources.

| Culture    | CDW <sup>a</sup><br>(g l <sup>-1</sup> ) | PHA%       | Composition (mol%) <sup>b</sup> |            |            |            |
|------------|--|------------|---------------------------------|------------|------------|------------|
|            |  |            | 3HHx                            | 3HO        | 3HD        | 3HDD       |
| Glucose    | 2.0 ± 1.2                                | 1.7 ± 0.5  | 0                               | 14.5 ± 1.1 | 69.1 ± 4.3 | 16.4 ± 3.8 |
| Glycerol   | 1.4 ± 0.1                                | 1.6 ± 0.2  | 0                               | 24.3 ± 1.4 | 56.9 ± 2.2 | 18.8 ± 0.8 |
| Citrate    | 1.8 ± 0.3                                | 0.4 ± 0.1  | 0                               | 0          | 54.3 ± 1.3 | 45.7 ± 1.3 |
| Fatty acid | 1.7 ± 0.3                                | 17.9 ± 1.1 | 8.6 ± 0.2                       | 60.0 ± 0.1 | 23.1 ± 0.3 | 8.3 ± 0.1  |

<sup>a</sup> CDW, cell dry weight.

<sup>b</sup> 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate, 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate.

sequence analysis indicated that the *glk* and *edd* genes were in the same operon, and *eda* and *zwf-1* genes were in another operon (13). This was consistent with our results since the expression levels of these two groups of genes shared similar patterns when cells were grown on different carbon sources (Fig. 4A and B). The expression levels of glucose ABC transporter genes were at least 6-fold higher when the cells were grown on glucose as compared to expression levels in cells grown on other carbon sources (Fig. 4A and B). This result is consistent with a previous study which measured carbon flux in *P. putida* KT2440 using <sup>13</sup>C (21).

**Fatty acid metabolism in *P. putida*** Fatty acids are activated with CoA by FadD for catabolism via the β-oxidation pathway (Fig. 1). There are three genes encoding putative FadD enzymes in the *P. putida* genomic database based on homology to other FadD proteins. A *fadD* knock-out mutant strain showed an 80-h long lag phase when grown on fatty acids as the sole carbon source (22), thus, *fadD* encodes the primary enzyme involved in β-oxidation, and functional equivalent *fadD* genes are induced when *fadD* is inactivated. Disruption of the *fadD2* gene had no effect on the growth of cells using fatty acid as the sole carbon source (22). In the current study, none of the *fadD* genes displayed high expression levels in cells grown on lauric acid compared to cells grown on other carbon sources (Fig. 6). These results suggested the expression levels of *fadD* were not induced by fatty acids, and the actual functions of *fadD2* and *fadDx* still need to be identified. Previous research suggested that the *fadBA* gene products were required for the catabolism of n-phenylalkanoic acids, whereas they can be replaced by similar enzymes for catabolism of aliphatic fatty acids (22). The *fadBx* and *fadAx* genes encode proteins that may replace the function of the FadAB enzyme. However, the gene expression levels of *fadBx* showed no significant differences ( $P > 0.05$ ) when cells were grown on different carbon sources, whereas the expression of *fadB* was induced by lauric acid (Fig. 6). Therefore *fadBx* and *fadAx* might not be involved in β-oxidation, or they may only be activated when the *fadB* and *fadA* genes are inactivated.

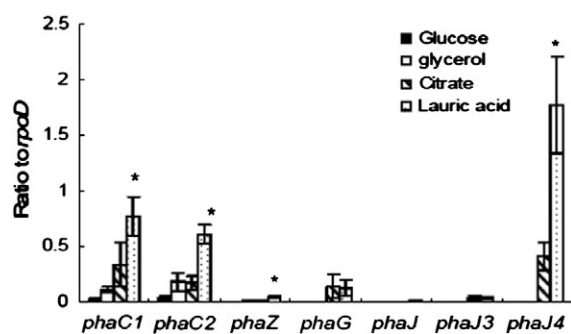


FIG. 7. Expression levels of genes related to PHA biosynthesis from cells grown on different carbon sources. Results are the averages ± standard deviations from three separate experiments. Asterisks indicate that the expression levels of cells grown on fatty acid have significant differences ( $P < 0.05$ ) from the other carbon sources.

**PHA metabolism in *P. putida*** Although PHA production has been well studied in pseudomonads, outside of studies on the PHA synthases and PHA depolymerases there is a surprising lack of information regarding the genes and enzymes involved in PHA metabolism, especially on the monomer-supply side. The current study has examined the effects of carbon feedstock on the expression of these genes.

Of the four *phaJ* genes that were identified in *P. aeruginosa*, *PhaJ1<sub>pa</sub>* was found to be specific for short-chain-length enoyl-CoAs of C4–C6, whereas the other three *PhaJs* were specific for medium-chain-length enoyl-CoAs of C6–C12 (14). Three *phaJ* genes were found in *P. putida* through a genomic database search, and no open reading frame was found that showed sequence similarity to *phaJ2<sub>pa</sub>*, which was consistent with previous research (23). The results showed that only the *phaJ4* gene showed higher expression levels when cells were grown on fatty acid and produced more PHAs (Fig. 7 and Table 2). Therefore, the gene *phaJ4* seems to be the only *phaJ* gene in *P. putida* involved in PHA biosynthesis. In order to confirm this conclusion, the knock-out mutant strain of *phaJ4* will need to be evaluated in future research. The results also showed that 3-hydroxyoctanoic acid (3HO) was the major monomer (Table 2), which was consistent with previous research that *phaJ4<sub>pa</sub>* had maximum activity for the C8 substrate (14). Fatty acid biosynthesis is the main route for the synthesis of 3-hydroxydecanoate, which is a substrate for PHA synthesis when cells are grown on unrelated carbon sources (24). It has been widely reported that PHAs can be synthesized under nutrient-limited conditions, such as nitrogen limitation (25). In the results presented here, the cells were grown on the media with a relatively high nitrogen concentration, so the production of PHAs was low in cells grown on unrelated carbon sources (glucose, glycerol and citrate) (Table 2). However, PHA production in cells grown on the related carbon source (lauric acid) was significantly higher. These results suggest that PHA production in cells grown on related carbon sources is not affected by nutrient limitations such as nitrogen concentration and could be of importance for the production of biomass and PHAs by *P. putida*.

In this study, expression levels of genes involved in metabolism of different carbon sources and PHA production were monitored. The results shed light on how these different metabolic pathways are regulated and which genes are actually involved with the metabolism of these various carbon feedstocks. New conclusions found in this study include: 1) Transcription of genes that encode transporter proteins is all highly regulated and thus likely represents first nodes of control for catabolism; 2) Not all genes encoding enzymes of a purported catabolic pathway for a specific carbon source are up regulated when the organism is grown on that particular carbon source (i.e. genes encoding enzymes for glycolysis in a cell grown on glucose or genes for beta-oxidation grown on fatty acids); 3) These genes that are strictly regulated by carbon source are potential target genes for metabolic engineering of the cells; 4) *P. putida* can produce large amounts of PHAs using fatty acid as carbon source without nitrogen limitation. The data generated by the research presented here will allow us to target specific genes and confirm their functions in the various metabolic pathways. This knowledge will also lead to the production of transgenic strains to enhance carbon utilization and PHA production.

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